Plant Physiol. (1978) 61, 779–784

Role of Lectins in Plant-Microorganism Interactions

II. DISTRIBUTION OF SOYBEAN LECTIN IN TISSUES OF *GLYCINE MAX* (L.) MERR.[1]

Received for publication June 19, 1977 and in revised form September 1, 1977

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ABSTRACT

Three different assay procedures have been used to quantitate the levels of soybean (*Glycine max* [L.] Merr.) lectin in various tissues of soybean plants. The assays used were a standard hemagglutination assay, a radioimmunoassay, and an isotope dilution assay. Most of the lectin in seeds was found in the cotyledons, but lectin was also detected in the embryo axis and the seed coat. Soybean lectin was present in all of the tissues of young seedlings, but decreased as the plants matured and was not detectable in plants older than 2 to 3 weeks. Soybean lectin isolated from seeds of several soybean varieties were identical when compared by several methods.

Symbiosis between bacteria of the genus *Rhizobium* and legume plants involves nodulation of the plant roots and biological fixation of atmospheric N_2. The host range for each species of *Rhizobium* is usually narrow, indicating that the *Rhizobium*-legume root symbiosis is a specific relationship. For example, *Rhizobium japonicum,* the soybean symbiont, does not infect green bean (*Phaseolus*) or clover (*Trifolium*), and, conversely, the green bean and clover symbionts do not infect soybean.

Several authors have suggested that the plant proteins known as lectins (phytohemmagglutinins) may be involved in the specific recognition of symbiotic rhizobia by legumes (2, 3, 10, 12, 30). Lectins have the capacity to bind to, or recognize, specific carbohydrate structures, and various species of legumes produce lectins with different binding specificities (18). Complex polysaccharides (*e.g.* lipopolysaccharides, exopolysaccharides) are present on the surfaces of Gram-negative bacteria, including *Rhizobium.* In many cases, the structures of such polysaccharides are known to be strain- or species-specific (21). Thus, the recognition of a symbiotic *Rhizobium* by a legume host may involve selective binding of the characteristic host plant lectin to a distinctive carbohydrate structure on the surface of the symbiont.

Bohlool and Schmidt (3) found that SBL[5] bound to 22 of 25 strains of the soybean symbiont, *R. japonicum,* but not to any strains of other rhizobial species. These results have been generally confirmed and extended (2). A similar pattern of lectin binding to symbiotic rhizobia, but not to nonsymbionts, has been observed for a lectin from white clover (*Trifolium repens*) (10). However, concanavalin A, a lectin from jackbean (*Canavalia ensiformis*), was reported to bind to all strains of rhizobia tested, irrespective of their ability to infect and nodulate jackbean (9).

If lectins are involved in recognition of rhizobial symbionts, the lectins must be present in or on the roots of the host plant. Yet there is little direct evidence that lectins are indeed present in these locations. The most common source of legume lectins is seeds (4, 29), although hemagglutinating activity, presumably due to lectins, has been detected in roots of soybean (3), and various tissues of several other legume species (6, 16). Howard *et al.* (14) and Rougé (26) dissected lentil (*Lens culinaris*) and pea (*Pisum sativum*) seeds, respectively, into seed coats, embryo axis, and cotyledons. High hemagglutinating activity was found in the cotyledon and embryo axis, but very little was detected in seed coats. Hemagglutination and immunodiffusion techniques have detected low levels of the seed agglutinins in roots, stems, and leaves of young lentil and pea seedlings, but not in nonseed tissues of older plants (14, 25, 26). Dazzo and Brill (8) have recently reported that *Rhizobium trifolii*-binding lectin can be eluted from the surface of intact white clover roots with a particular sugar hapten. This lectin appears to be localized at or near the tips of the root hairs.

As part of our investigation of the involvement of lectins in plant-microorganism interactions, we have developed two techniques to measure specific lectins in various plant extracts quantitatively. We now report on the use of these techniques to determine the quantity of soybean seed lectin in various tissues of soybean seedlings of different ages.

MATERIALS AND METHODS

Preparation of Flour from Whole Seeds and Seed Tissues. Flours were prepared from whole seeds of *Glycine max* (L.) Merr. vars. Acme, Beeson, and Wayne. Seed samples were ground to 40 mesh with a Wiley mill and defatted with petroleum ether. Commercial soybean flour (Soya Fluff 200W, Central Soya Chemurgy, Chicago, Ill.) was defatted as above, and routinely used as a source of SBL.

To procure material for analyzing the distribution of SBL in soybean seeds (var. Beeson), seeds were soaked for 5 min in warm water. The swollen seed coats were then removed, and the embryos were dissected into the cotyledons and embryo axis. The pooled samples of embryo axis, of seed coats, and of cotyledons were dried in a forced air oven at 70 C. This drying procedure does not measurably inactivate SBL. Cotyledons and seed coats were ground to 40 mesh and defatted as above. Embryo samples, because of their small size, were defatted directly, and not passed through the Wiley mill. Defatted samples of embryo axis tissue

[1] Supported in part by National Science Foundation Grants BMS 75-17710 and BMS 76-00913.

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[5] Abbreviations: SBL: soybean lectin; PBS: phosphate-buffered saline.

were pulverized with a mortar and pestle just before analysis.

The contribution of each seed tissue to seed weight was estimated by separating two lots of 50 seeds each into seed coats, cotyledons, and embryo axes. The tissues were dried at 70 C for 96 hr, and dry weights were recorded.

Growth of Soybean Seedlings. For isotope dilution assays, undamaged soybean seeds (var. Beeson) were planted in pans (10 × 12 × 2 cm deep) containing Vermiculite, irrigated with Hoagland (13) nutrient solution, and maintained in a growth chamber at 25 C and about 7,500 *lux* from fluorescent and incandescent light sources (16-hr photoperiod). Plants assayed for lectin by hemagglutination and radioimmunoassays were grown under similar conditions except the temperature was maintained at 32 C and 27 C during the day and night cycles, respectively. Under these conditions the seedlings emerged on the 3rd day after planting. The Vermiculite was kept moist by addition of distilled H_2O as needed after planting.

Healthy seedlings were harvested on various days after planting by carefully removing their roots from the Vermiculite and washing them in a gentle stream of water. Roots, stems, and cotyledons were then excised, blotted dry, and weighed immediately. Tissue samples were stored frozen until used.

Purification of SBL. SBL was purified by affinity chromatography using the procedure described by Allen and Neuberger (1). The method of Lowry *et al.* (20) was used to assay for protein content with BSA (Sigma) as a standard. The homogeneity of SBL preparations was determined by electrophoresis using 7.5% polyacrylamide disc gels (0.5 × 7.5 cm) and the acidic buffer system of Reisfeld *et al.* (23). Gels were electrophoresed at 3 mamp/gel until the tracking dye was less than 1 cm from the end of the tubes. Staining was done with Coomassie brilliant blue R-250.

Radiolabeling of SBL. Tritium-labeled SBL for isotope dilution experiments was prepared by oxidation with sodium periodate followed by reduction with potassium ^{3}H-borohydride (24.4 mCi/mmol, Schwarz/Mann) according to the procedure of Lotan *et al.* (19). ^{3}H-SBL was purified by gel filtration chromatography on a column of Bio-Gel A-0.5m (2.5 × 30 cm) with 0.19% NaCl as eluant and then by affinity chromatography as described above. Recovery of unaggregated ^{3}H-SBL was 82%.

Samples were counted with a Nuclear-Chicago liquid scintillation counter using Aquasol 2 (New England Nuclear) as a scintillation fluid. Counting efficiency was about 43%, and specific radioactivity of the ^{3}H-SBL was 1.32×10^7 cpm/mg. Prior to use in isotope dilution experiments, the ^{3}H-SBL was diluted with unlabeled SBL to a more appropriate specific radioactivity.

Iodine-labeled SBL (^{125}I-SBL) for radioimmunoassays was prepared using the chloramine-T procedure as described by Sela *et al.* (27). The iodinated SBL was purified twice by affinity chromatography before use in the radioimmunoassay. The specific radioactivity was 2×10^8 cpm/mg.

Quantitation of SBL in Plant Materials by Isotope Dilution. The amounts of SBL in root and cotyledon samples from soybean seedlings were determined as follows. The tissue sample was added to PBS (0.43 g of KH_2PO_4, 1.48 g of Na_2HPO_4, 7.20 g of NaCl/liter, pH 7.2) containing 0.05 M ascorbate in a Waring Blendor. ^{3}H-SBL (45–90 μg, specific radioactivity 2.2×10^6 cpm/mg) was introduced, and several drops of octanol were added to retard foaming. The sample was ground to a fine suspension and centrifuged at 9,200*g* for 15 min. The supernatant solution was filtered through glass fiber paper and stirred at 4 C with 9 ml of affinity beads. After 2 hr, the affinity beads were collected in a sintered glass funnel, rinsed carefully with PBS, and poured into a column (0.9 × 15 cm). The column was eluted with PBS, and the eluant was monitored by *A* at 280 nm (A_{280}). When the A_{280} stabilized at a base-line value, elution with 100 mM galactose or 1 mM N-acetyl-D-galactosamine in PBS was initiated. The eluted SBL was monitored as an A_{280} peak and was collected as a single fraction. The specific radioactivity of the recovered SBL from each sample was then measured by protein determination and scintillation counting, and an aliquot of each sample was electrophoresed to monitor the identity and homogeneity of the purified protein. The recovery of radiolabel varied from 55 to 100%.

Flours from seeds (50 g/sample) and seed tissues (2 g/sample) were suspended in PBS (20 ml/g) and ^{3}H-SBL was added. After stirring for 1 hr at room temperature, the suspensions were centrifuged and analyzed as for the tissue sample extracts above. The extracts from 2 g of flour were incubated with 9 ml of affinity beads, whereas extracts from 50 g of flour were incubated with 50 ml of beads.

Quantitation of SBL Levels Using Radioimmunoassay. Antiserum against SBL was prepared in rabbits by intramuscular injections of purified SBL in complete Freund's adjuvant. Antiserum against rabbit IgG was prepared in a goat by injection of purified rabbit IgG in complete Freund's adjuvant.

Several series of experiments were carried out to determine the appropriate dilutions of ^{125}I-SBL and rabbit antiserum to be used in the assay, as well as the optimum time for each incubation. The ^{125}I-SBL was diluted in PBS containing 0.25% BSA to a final concentration of 40,000 cpm/ml. The 50-μl aliquot of this solution used in the assay contained approximately 20 ng of SBL. The same batch of rabbit antiserum was used for all of the experiments described here. This antiserum was diluted in PBS such that the aliquot of antiserum used would precipitate approximately 70 to 90% of the ^{125}I-SBL. This was typically a dilution of 35,000-fold.

A typical radioimmunoassay consisted of 250 μl of properly diluted rabbit anti-SBL serum, 50 μl of ^{125}I-SBL, and 100 μl of a sample containing 0.1 to 1.0 μg of SBL. This mixture was incubated for 1 hr at 4 C, followed by the addition of 20 μl of undiluted goat anti-rabbit IgG. This mixture was incubated 24 hr at 5 C. The immunoprecipitate was collected by centrifugation for 20 min at 3000 rpm in a nonrefrigerated table top centrifuge. The amount of ^{125}I-SBL in the supernatant solution and the precipitate was determined with a Nuclear-Chicago gamma counter. The percentage of ^{125}I-SBL in the immunoprecipitate was calculated from these numbers. Each sample was assayed in duplicate.

Weighed tissue samples to be extracted for radioimmunoassays were cooled to dry ice temperatures and ground into a fine powder in a mortar kept on dry ice. This powder was extracted for 1 to 2 min with 10 volumes of PBS containing 0.5% galactose. The resulting mixture was filtered through a glass fiber filter. Phenylmethane sulfonyl fluoride was added to each filtrate to a final concentration of 1 mg/ml in order to inhibit protease activity. The filtrate was stored frozen until it was assayed for protein content and SBL content.

For every sample, a control for nonspecific binding was performed using PBS instead of the rabbit antiserum. The percentage of ^{125}I-SBL bound in this control (less than 5% if freshly prepared ^{125}I-SBL was used) was subtracted from the experimental sample. A standard curve was prepared for each experiment using increasing dilutions of purified SBL. One such standard curve is shown in Figure 1. The standard curve from each experiment was used to convert the value for per cent ^{125}I bound for an unknown sample into μg of SBL/ml.

Quantitation of SBL Levels Using Hemagglutination. Hemagglutination assays were carried out with trypsinized rabbit red blood cells (Gibco) using the microtiter plate assay (11). In some cases hemagglutination was detected by observation of the red blood cells under a microscope. This assay gave the same results as the microtiter plate assay but was less sensitive and less convenient. With every series of unknown samples, a standard curve was also determined. This allowed a conversion from hemagglutination titer (the reciprocal of the last dilution which gave hemagglutination) to the concentration of lectin. This assay is very sensitive and can detect levels of lectin as low as 10 ng/ml. There is, however, difficulty in reproducibly determining the titer of a

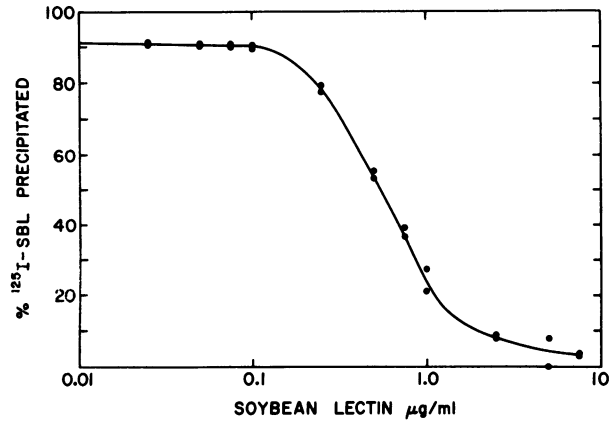

FIG. 1. Typical standard curve for the radioimmunoassay. Percentage of ^{125}I-SBL precipitated is plotted as a function of increasing quantities of purified SBL. Percentage of ^{125}I-SBL precipitated was calculated by measuring the amount of radioactivity in the precipitate and the supernatant.

sample, and errors of 200 to 400% in the levels of lectin determined are possible.

Samples for hemagglutination assays were prepared as for the radioimmunoassays, dialyzed, and stored frozen. Each data point represents the mean of the SBL levels from four to eight plants. Tissues from one plant on each of the sample days were extracted using PBS which contained 1 μg/ml of ^{3}H-SBL (added to a level of 1 μg ^{3}H-SBL/g fresh wt). The recovery of tritium cpm in the final extract was determined and the final concentration of lectin/g of tissue was corrected using the recovery factor. The recoveries ranged from 60 to 100%.

RESULTS

SBL in Seeds and Seed Parts. The distribution of SBL in embryo axes, cotyledons, and seed coats from soybean seeds is shown in Table I. Although SBL was present in all parts of the seed, the cotyledons contained disproportionately large concentrations of the lectin.

Three criteria were used to evaluate whether the SBL from the three varieties and the commercial source were the same. First, the electrophoretic mobilities of all four preparations of SBL were found to be identical using polyacrylamide gel electrophoresis at pH 4.3. The electrophoretic patterns of SBL from Beeson, Acme, and the commercial flour (Wayne not examined) were also identical after polyacrylamide gel electrophoresis in the presence of SDS (17). The second criterion was reactivity with rabbit anti-SBL serum. The SBL from Beeson, Acme, Wayne, and the commercial flour all reacted equally well with the rabbit antiserum prepared against SBL from the commercial flour. The third criterion was to compare the binding activity of each SBL toward the receptors on *R. japonicum*, as described previously (2). Aliquots of a commercial flour ^{3}H-SBL stock solution (1.32×10^7 cpm/mg) were diluted approximately 50-fold with unlabeled SBL from each of the three soybean varieties and from commercial flour to give new solutions with specific radioactivities of 2.8×10^5 cpm/mg of SBL. The binding activities of the new solutions were then assayed with washed, living cells of *R. japonicum* strain 3I1b 138. When 1×10^9 bacterial cells were mixed with 114 μg of SBL from the different stocks in 1 ml of PBS, the percentage of SBL bound to the bacteria was the same (93–94%).

Levels of SBL in Tissues of Young Plants. The quantity of SBL in various tissues of young plants was determined using the three different assays described. The levels of SBL in the cotyledons were highest at the time of planting (Fig. 2), but decreased rather steadily until abscission. This general pattern was observed using each of the three different assays (Fig. 2). Between 0.1 and 1 μg of SBL/g fresh wt could be detected by the hemagglutination and radioimmunoassays at the time of cotyledon abscission (16–18 days).

Table I. Distribution of SBL in seeds of Glycine max var. Beeson

SBL concentrations given are the mean ± SD of three experiments. Values presented in this table were determined using the isotope dilution assay.

Tissue	% Dry Wt of Seed	SBL (mg/g defatted flour)
Cotyledon	92.0	9.14 ± 0.14
Embryo axis	1.8	1.39 ± 0.12
Seed Coat	6.2	1.81 ± 0.12
Whole Seed	100.0	7.99 ± 0.22

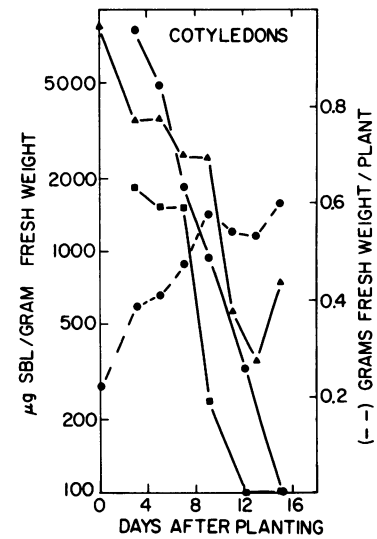

FIG. 2. Levels of SBL in cotyledons of young plants. Soybean cotyledon tissues from seedlings of various ages were extracted. Extracts were assayed for SBL by the isotope dilution assay (▲), the hemagglutination assay (■), and the radioimmunoassay (●). Each data point for the isotope dilution assay is the average of two separate determinations of SBL in extracts of pooled cotyledon tissues from several plants. Data points for the hemagglutination assay and the radioimmunoassay are the average from eight plants, each of which was analyzed separately. A typical growth curve of the seedlings is shown (●- - -●).

Other tissues of young plants were also examined for the presence of SBL. The results obtained using the radioimmunoassay and hemagglutination assay are shown in Figure 3. Stems, primary roots, secondary roots, and leaves were examined separately. In each case, readily detectable levels of SBL were present in very young tissues. As the plants aged, the levels of lectin decreased until there was no detectable lectin in the plants (15–16 days after planting). Plants up to 30 days old were analyzed, and no lectin was found by either method. The limit of detectability for both assays was about 0.1 μg of SBL/g fresh wt of plant tissue. For every time point, ^{3}H-SBL could be recovered from controls containing exogenous ^{3}H-SBL. This indicates that the failure to detect SBL in some samples was not due to its absorption onto insoluble residues during the extraction procedures.

When the levels of SBL in root tissues were measured with the isotope dilution assay, a quantitative discrepancy with the data from hemagglutination and radioimmunoassays was observed (Figure 4). The level of SBL by isotope dilution was approximately 200 μg/g fresh wt in roots of 3-day-old seedlings, and decreased gradually thereafter to approximately 60 μg/g fresh wt 13 to 15 days after planting. In contrast, SBL levels by hemagglutination and radioimmunoassay were approximately 50 μg/g fresh wt 3 to 4 days after planting and less than 1 μg/g fresh wt 13 to 15 days after planting.

Disparity in the data from root tissues obtained by the different assay techniques was evaluated by performing two types of pro-

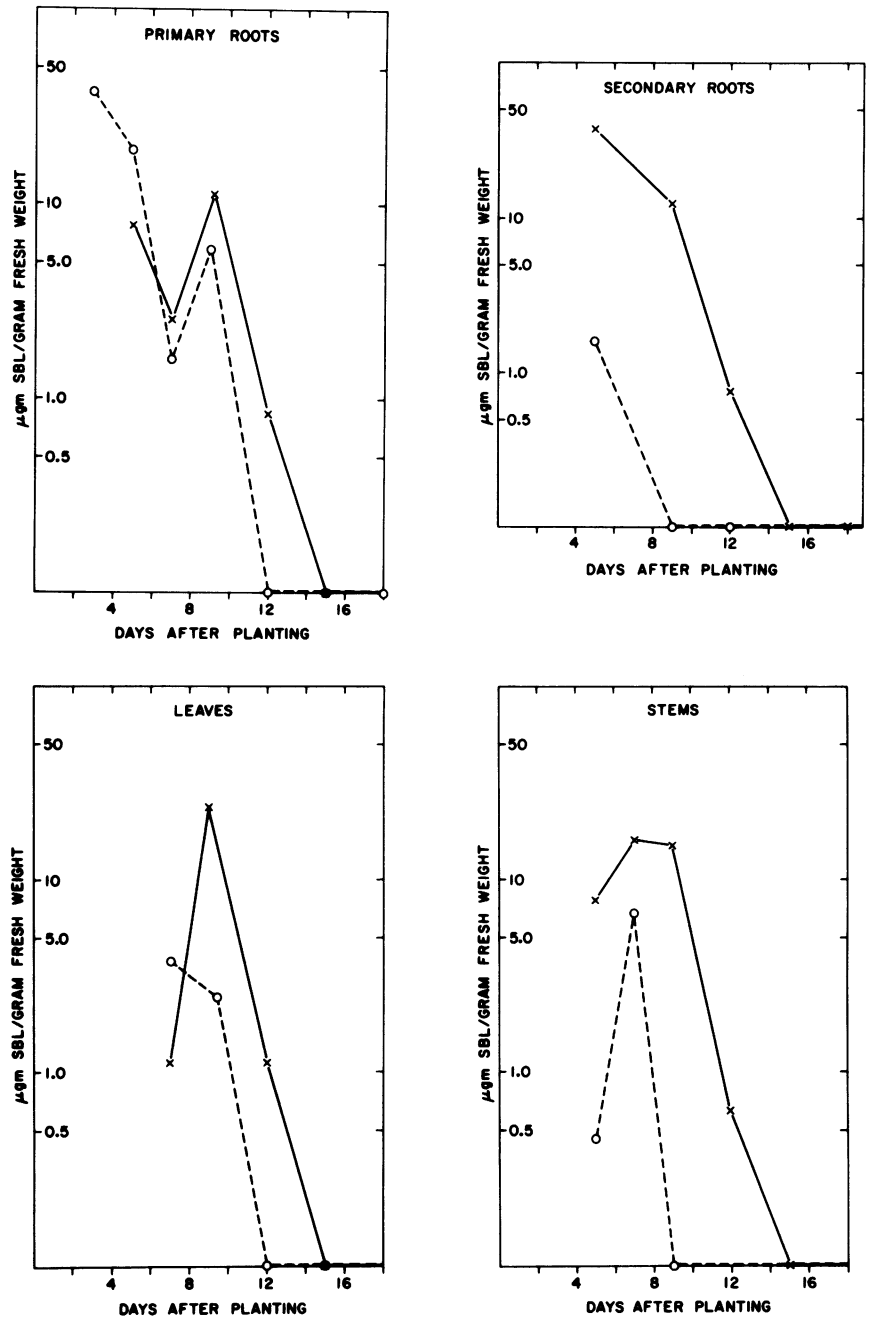

FIG. 3. Levels of SBL in tissues of young soybean plants. Plants were grown and tissues were extracted as described under "Materials and Methods." Extracts were assayed for SBL using the radioimmunoassay (×——×) and the hemagglutination assay (O- - -O). Each data point is the average of four to eight plants each of which was analyzed separately.

cedural controls. In the first, root tissues from plants 10 to 12 days old were extracted as described for the isotope dilution assay. The tissues (4.3 g) were extracted in 43 ml of buffer containing 8.6 μg of ^{3}H-SBL (specific radioactivity 7.2 × 10^6 cpm/mg). A small aliquot of the crude extract was retained for radioimmunoassay, and the remainder was purified by affinity chromatography as in the isotope dilution experiments. The specific radioactivity of the repurified SBL was determined, and using this value, the amount of SBL was calculated to be 153 μg/g fresh wt, a value in close agreement with earlier isotope dilution data. However, the radioimmunoassay indicated that there were 7 μg of SBL in the crude extract and 4 μg of SBL in the purified extract from the affinity column. These amounts can be accounted for by the original 8.6 μg of ^{3}H-SBL added, and indicate that the material giving the apparent isotope dilution in the root extracts is not the seed SBL.

In a second set of experiments, small amounts of defatted soybean flour from cv. Beeson were analyzed by the isotope dilution procedure in the presence or absence of large amounts of pinto bean flour, which contains no SBL. Experimental recoveries of SBL were compared with recoveries expected on the basis of earlier experiments using large amounts of Beeson flour. It was found that the isotope dilution procedure gives erroneously high values for SBL when less than 2.5 mg of the lectin is recovered. The addition of pinto bean flour has no significant effect if this threshold quantity of SBL is present. We interpret this to mean that small quantities of Lowry-positive contaminating substances, which are not detected in polyacrylamide gels, adhere to the affinity beads and elute with SBL. Based on comparisons with data from the other procedures, the isotope dilution technique is

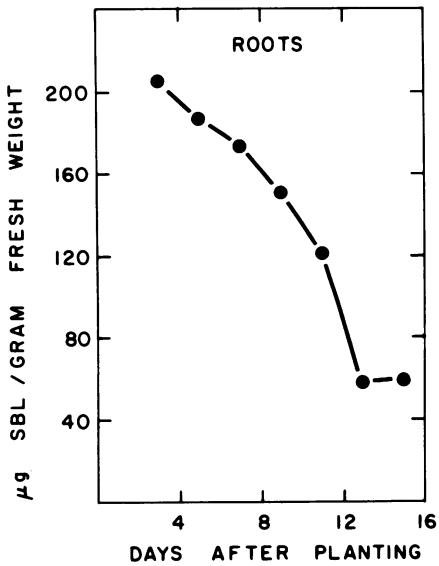

FIG. 4. Isotope dilution assay of SBL in roots. Plants were grown and root tissues extracted as described under "Materials and Methods" for isotope dilution assays. Each data point is the average of two separate determinations of SBL in extracts of pooled root tissue from several plants.

suitable for analysis of tissues rich in SBL (cotyledons, seeds), but may not be suitable for plant parts such as roots and vegetative shoots which contain little SBL.

DISCUSSION

As expected, the greatest quantities of SBL were found in the cotyledons (Table I); however, there are appreciable levels of SBL in both the seed coat and the embryo axis. This is in contrast to lentil seeds and pea seeds, which have appreciable concentrations of lectin in the embryos, but very low or negligible levels of lectin in the seed coat (14, 24, 26). Krüpe and Ensgraber (16) examined seeds of several different plants and found high levels of lectin in the embryo axis of some plants, while the other plants had little or no lectin in the embryo axis.

Seeds of three varieties of soybean (Acme, Beeson, and Wayne) all contained SBL; SBL in preparations from these varieties coelectrophoresed in polyacrylamide gels, reacted equally with rabbit anti-SBL antiserum, and could not be differentiated on the basis of binding to *R. japonicum*. A preliminary report indicates that two isolines of soybean var. Harosoy also contain SBL (5).

The pattern of declining amounts of SBL in soybean cotyledons (Fig. 2) is similar to that observed by Rougé (26) for the lectins in pea cotyledons. The lectins in both lentil and *Phaseolus vulgaris* cotyledons drop below detectable levels prior to abscission (22, 24).

The roots, stems, and leaves of young soybean plants all contain detectable levels of lectin (Fig. 3), albeit in much lower amounts than in the cotyledons. The levels of SBL in these tissues are highest at the early ages, then decrease as the plants mature. After 14 to 16 days, the lectin is below the level of 0.1 μg/g fresh wt, the limit of detectability in our assays. Assays of plant tissues from older plants have shown that the lectin is absent or remains below detectable levels in all vegetative tissues. This pattern is similar to that reported for lentil and *P. vulgaris* (14, 22, 25). Mialonier *et al.* (22) reported that extracts from bean leaf tissues contained an immunologically cross-reactive protein that lacked hemagglutinating activity. In soybean plants, we found no evidence for a protein in extracts of leaf tissue which cross-reacted with the antiserum directed against SBL.

The presence of SBL in the tissues of young plants raises the interesting question of the origin of this SBL. The total amount of SBL in the embryo axis is too small to account for the amount seen in the young plant tissues. SBL in these tissues must either be synthesized within each tissue or be transported there from the cotyledons. The similar patterns of lectin levels as a function of age in the various tissues suggest that the cotyledons may be the source of the SBL present in the other tissues. In this regard, it is interesting to note that Kauss and Ziegler (15) have reported the presence of hemagglutinating lectins in the sieve-tube sap of *Robinia pseudoacacia* L.

The fact that soybean seed lectin cannot be detected in root tissue extracts from older plants raises questions concerning the possible involvement of SBL in the recognition of the nitrogen-fixing symbiont, *R. japonicum*. In reviewing the susceptibility of older plants to infection by rhizobia, Dart (7) points out that many plants are more susceptible to nodulation when inoculation is delayed from 5 to 25 days. Skrdleta (28) examined the nodulation of older soybean plants and found the highest rate of nodulation occurred in plants infected 2 to 4 weeks after sowing. He also notes that younger portions of the roots are infected during a delayed inoculation. Thus, nodulation of soybean occurs in older plants at times when our results indicate that SBL can no longer be detected in the roots of these plants.

There are several possible interpretations of these results. First, it is possible that SBL has nothing to do with the recognition of *R. japonicum* or initiation of the infection process. Second, it is possible that SBL in older plants is synthesized or located in very restricted areas of the roots (*e.g.* the tips of growing root hairs). Whereas the local concentration of SBL in these areas may be appreciable, the total amount of SBL could be below the limits of detection by our assays when the entire root tissue is extracted. This possibility is currently under investigation by means of fluorescent antibody localization of SBL in root tissues. A third possibility is that SBL from the seeds gradually becomes incorporated into, or associated with, membranes or cell walls or other structures of root cells such that the lectin is not rendered soluble by simple extraction with buffer. A fourth possibility, one which needs to be considered in regard to all investigations of lectins and their possible role in rhizobial infection, is that there may be a lectin present in the roots of the host which is separate and distinct from the seed lectin. Preliminary investigations (Keegstra, unpublished data) have indicated that a hemagglutinating lectin from soybean roots, with a binding specificity similar to, but readily distinguishable from that of the seed lectin, can be extracted from crude root membrane preparations with buffer containing the detergent Triton X-100. Further experiments are in progress to purify and characterize this lectin with regard to its *Rhizobium*-binding properties and its biochemical relationship, if any, to the seed lectin.

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